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PATENT SPECIFICATION

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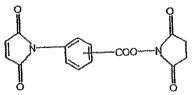
(19)

(54) REAGENT SUITABLE FOR ENZYME IMMUNO ASSAY

(71) We, DAINIPPON PHARMACEUTICAL CO. LTD., a body corporate organised under the laws of Japan of 25 Dosho-machi 3-chome, Higashi-ku, Osaka-shi, Osaka-fu, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

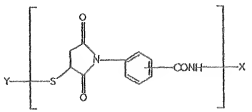
The present invention relates to a reagent suitable for an enzyme immunoassay. More particularly, the invention relates to the use of a maleimidobenzoic acid N-hydroxysuccinimide ester (hereinafter referred to as "MBS") of the formula (I):



(I)

as a binding agent for binding an enzyme and an antigen.

The invention provides an enzyme-labelled antigen of the formula (II):



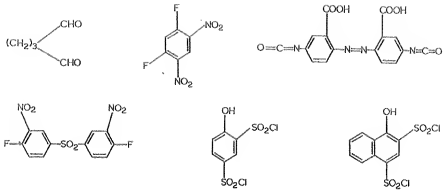
(II)

wherein X and Y are different and are each an enzyme or an antigen; an enzyme immunoassay using the above enzyme-labelled antigen; and a kit containing the enzyme-labelled antigen.

The compounds of formula (I) are described and claimed in our co-pending Application No. 48612/78 (serial no. 1,570,533) divided from the present application.

In the present specification, by enzyme immunoassay is meant any assay in which an enzyme-labelled antigen is used for an antigen-antibody reaction.

The enzyme immunoassay is generally carried out by subjecting an enzyme-labelled antigen, an unlabelled antigen (i.e. the substance to be measured) and an antibody to a competitive antigen-antibody reaction in a buffer solution, separating the enzyme-labelled antigen bound to the antibody and the free enzyme-labelled antigen (i.e. the enzyme-labelled antigen to which no antibody was bound), and determining the amount of the unlabelled antigen (i.e. the substance to be measured) from the enzyme activity on either the antibody-bound enzyme-labelled antigen or the free enzyme labelled antigen. This method has been described in detail in various literature references, for instance, in U.S. Patent Specifications Nos. 3,654,090, 3,839,153 and 3,850,752.



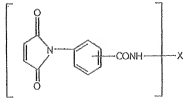
These binding agents contain the same two functional groups, and hence, when these reagents are used for binding the enzyme and the antigen, unfavourable products such as an antigen-antigen and/or an enzyme-enzyme complex are formed in addition to the desired enzyme-antigen complex (i.e. the enzyme-labelled antigen). Accordingly, it is difficult to isolate only the desired enzyme-labelled antigen from the mixture of these three products. In particular, the presence of the enzyme-enzyme complex causes disturbance in the enzyme immunoassay.

Under the circumstances, we have sought an improved binding agent which can selectively bind the enzyme on one hand and the antigen on the other hand.

As a result, we have found that the MBS of the formula (I) can selectively bind the enzyme and the antigen under very mild conditions, and further that the enzyme-labelled antigen prepared by means of the MBS can be favourably used for the enzyme immunoassay.

The present invention provides a novel compound (I) suitable for binding an enzyme and an antigen. The enzyme and the antigen can be selectively bound by using the MBS of the formula (I), and hence, only the desired enzyme-labelled antigen can be prepared. Some compounds which are structurally similar to the MBS have been described in Helvetica Chimica Acta, Vol. 58, pages 531—541 (1975), but this reference does not describe the application of these compounds to the enzyme immunoassay.

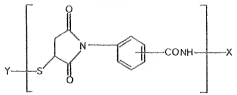
The binding of the enzyme and the antigen comprises the steps of (i) reacting an antigen or enzyme, which contains an amino group but does not contain any thiol group, with the ester moiety of MBS to form a bound compound of the formula (II):



(II)

wherein X is an antigen or an enzyme, and

(ii) reacting the resulting bound compound (II) with an enzyme or antigen which contains a thiol group and may optionally contain an amino group, whereby the maleimido moiety of the MBS in the bound compound (II) is subjected to the addition reaction with the enzyme or antigen to form the enzyme-labelled antigen of the formula (III)



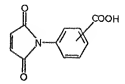
(III)

wherein X and Y are different and are each an enzyme of an antigen.

Thus MBS is an excellent bifunctional binding agent having a high selectivity and can bind the enzyme and the antigen through two-step reactions under very mild conditions, which are substantially different from the manner in which conventional binding agents act.

The MBS of formula (I) can exist in the form of three isomers, viz. the ortho-substituted compound (hereinafter referred to as "*o*-MBS"), the meta-substituted compound (hereinafter referred to as "*m*-MBS") and the para-substituted compound (hereinafter referred to as "*p*-MBS"). Among these compounds, the most suitable is *m*-MBS.

The MBS of the formula (I) is a novel compound and may be easily prepared by reacting a maleimidobenzoic acid of the formula:



with N-hydroxysuccinimide of the formula:

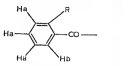
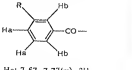
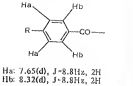
in an organic solvent (e.g. tetrahydrofuran, dioxane, benzene or acetone) in the presence of a dehydrating agent (e.g. dicyclohexylcarbodiimide), preferably at room temperature for 2 to 3 hours.

The present invention is illustrated by the following Example and Reference Example, but is not limited thereto.

Example.

Preparation of *o*-,*m*- and *p*-MBS

To a solution of *o*-, *m*- or *p*-maleimidobenzoic acid (217 mg) in tetrahydrofuran (30 ml) are added N-hydroxysuccinimide (130 mg) and dicyclohexylcarbodiimide (224 mg). The mixture is stirred at room temperature for 2 hours, and the precipitated N,N'-dicyclohexylurea is filtered off. The filtrate is concentrated under reduced pressure. The residue thus obtained is purified by silica gel column chromatography (elute: chloroform), and then recrystallized from ether-dichloromethane to give *o*-, *m*- or *p*-MBS having the properties shown in the following Table 1.

	Melting point (°C)	Elementary Analysis $C_{18}H_{14}N_2O_6$ Calculated (%) / Found (%)			IR (KBr) νmax (cm^{-1})	NMR (60MHz, $CDCl_3$) δ-value		
						$-CH_2CH_2-$	$-CH\cdot CH-$	Hydrogen in benzene ring
O-MBS	125 ~ 128	C 57.33 57.29	H 3.21 2.95	N 8.92 8.85	3100 (=C–H), 1770, 1731, 1720 (Maleimide, Succinimide, –COON=), 1559, 1495, 1390, 1202, 1065 (–COON=), 986, 842, 701	2.82(s) 4H	6.86(s) 2H	 Ha: 7.28–7.95(m), 3H Hb: 8.13–8.37(m), 1H
m-MBS	182 ~ 185	C 57.33 57.22	H 3.21 2.98	N 8.92 8.72	3110 (=C–H), 1773, 1738, 1712 Maleimide, Succinimide, –COON=), 1487, 1446, 1392, 1204, 1075 (–COON=), 830, 699	2.90(s) 4H	6.89(s) 2H	 Ha: 7.57–7.77(m), 2H Hb: 8.03–8.23(m), 2H
p-MBS	198 ~ 200	C 57.33 56.90	H 3.21 3.18	N 8.92 8.65	3095 (=C–H), 1774, 1740, 1718 (Maleimide, Succinimide, –COON=), 1605, 1513, 1375, 1208, 1075 (–COON=), 1002, 832, 699	2.90(s) 4H	6.90(s) 2H	 Ha: 7.65(d), J=8.8Hz, 2H Hb: 8.36(d), J=8.8Hz, 2H

R: maleimide

Among two functional groups in these MBS compounds, the maleimido moiety is considered to be unstable, while the ester moiety is considered to be less reactive for the antigen or enzyme. The stability and reactivity of the MBS compounds were tested. The results are shown in the following Table 2 and 3, respectively.

TABLE 2 Stability of MBS

Test compound	Incubation time				
	30 minutes				20 minutes
	pH range 5.0	6.0	7.0	8.0	7.5
o-MBS	3.1	6.2	21.4	69.0	18.8
m-MBS	2.9	2.5	7.1	43.8	9.4
p-MBS	3.8	6.6	32.0	52.0	37.5

[Remark]: The numeral in the above table means percentage of decomposed maleimido group of MBS, when a solution of the test compound (10 mmol) in tetrahydrofuran (20 μl) is mixed well with 0.5 of a 0.005 M phosphate buffer (pH: 6.0, 7.0, 7.5 or 8.0) or a 0.05 M citrate buffer (pH: 5.0), and the mixture is incubated for 20 or 30 minutes.

TABLE 3 Reactivity of MBS

Test compound	Reactant: lysine
o-MBS	32.6
m-MBS	41.2
p-MBS	27.5

[Remark]: The numeral in the above table means the acylation percentage of the reactant: lysine with MBS, when a solution of the test compound (1 mmol) in tetrahydrofuran (10 μl) is mixed with 0.1 ml of a 0.1 M lysine — 0.05 M phosphate buffer (pH: 7.5), and mixture is reacted at 30°C for 20 minutes.

Reference Example.

Measurement of insulin

a) Binding of *m*-MBS to insulin

to a 0.05 M phosphate buffer (pH: 7.0, 1 ml) containing pig insulin [made by Schwarz Mann G.m.b.H., 6 mg (1 μ mol), 25.5 U/mg] is added a solution of *m*-MBS (1.2 μ mol, i.e. 5 mg/ml) in tetrahydrofuran (75 μ l), and the mixture is allowed to stand at room temperature for 30 minutes, during which it is occasionally stirred. To the resulting mixture is added to 1 M citrate phosphate buffer (pH: 5.0, 1 ml), and the resulting precipitates are separated by centrifuging (800 × g, 15 minutes). The precipitates thus obtained are washed twice with a 0.01 M citrate buffer (pH: 5.3, 2 ml) and dried under reduced pressure to give [*m*-MBS]-[insulin] bound product (5.5 mg).

b) Binding of [*m*-MBS]-[insulin] product to β-D-galactosidase

To a 0.05 M phosphate buffer (pH: 7.0, 1 ml) containing β-D-galactosidase of *Escherichia coli* [made by Boehringer Mannheim G.m.b.H. in West Germany, 0.93

nmol (500 μ g)] is added to a 0.05 M phosphate buffer (pH: 7.0, 0.15 ml) containing the [*m*-MBS]-[insulin] bound product (151 μ g, i.e. maleimido content: 3.6 nmol) obtained in the above (a), and the mixture is allowed to stand at room temperature for 2 hours.

The resulting mixture is passed through a Sepharose (trade mark) 6B column (1.3 x 33 cm, made by Pharmacia Fine Chemicals in Sweden), which is eluted with a 0.1 M NaCl — 0.02 M phosphate buffer (pH: 7.0) and the desired [β-D-galactosidase]-[*m*-MBS]-[insulin] product. Yield: about 80% calculated from the enzymatic activity thereof. The molar ratio of insulin and β-D-galactosidase of the product is substantially 1 : 1.8.

c) Measurement of insulin

The main fraction of the [β-D-galactosidase]-[*m*-MBS]-[insulin] bound product (i.e. enzyme-labelled antigen) obtained in b) above is diluted 200 times with water. The diluted enzyme-labelled antigen (10 μ l) is mixed with a 0.1% rabbit serum albumin — 1 mM $MgCl_2$ — 0.1 M NaCl 0.1% NaN_3 — 0.02 M phosphate buffer (pH: 7.0) (0.2 ml) containing insulin (i.e. unlabelled antigen, 0 — 20 μ U) and anti-pig insulin guinea pig antiserum (made by Dainabot Radioisotope Lab., Ltd. in Japan, 50 μ) (i.e. the first antibody). The mixture is allowed to stand at 4°C for 16 hours, and anti-rabbit 7*s*-γ-globulin anti-guinea pig serum (i.e. the second antibody, 10 μ l) is added. The mixture is further allowed to stand at 4°C for 8 hours and then subjected to centrifuging (800 x g, 15 minutes). The activity of β-D-galactosidase in the supernatant fluid or the precipitates thus obtained is measured by the method mentioned in the following part (d), from which a standard calibration curve as shown in the accompanying drawing is obtained. Accordingly, it is clear that the insulin of 0.5 to 20 μ U can be measured by this enzyme immunoassay.

d) Measurement of the activity of β-D-galactosidase

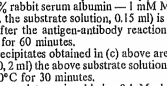
To a solution of a 0.1 mM 4-methylumbellifery-β-D-galactoside — 0.2 M sodium phosphate — 0.1% rabbit serum albumin — 1 mM $MgCl_2$ — 0.1 M NaCl — 0.1% NaN_3 (pH: 7.0) (i.e. the substrate solution, 0.15 ml) is added the supernatant fluid (50 μ l) obtained after the antigen-antibody reaction in (c) above, and the mixture is kept at 30°C for 60 minutes.

Alternatively, the precipitates obtained in (c) above are washed with a 0.05 M phosphate buffer (pH: 7.0, 2 ml) the above substrate solution (0.15 ml) is added, and the mixture is kept at 30°C for 30 minutes.

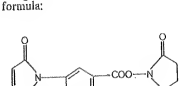
To each of the above mixtures is added a 0.1 M glycine — NaOH buffer solution (pH: 10.3, 2.5 ml) in order to stop the reaction. The 4-methylumbelliferone produced in the reaction mixture in proportion to the activity of enzyme-labelled antigen is measured by a MPF 4 type spectro-fluorometer (made by Hitachi, Ltd. in Japan) at exeitation wave length: 365 nm and emittion wave length: 448 nm.

WHAT WE CLAIM IS:—

1. A maleimidobenzoic acid N-hydroxysuccinimide ester of the formula:

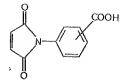


2. A compound according to claim 1, wherein the compound is a meta-position isomer of the following formula:



3. A compound as claimed in Claim 1, substantially as hereinbefore described with reference to the Example.

4. A process for preparing a compound as claimed in Claim 1, which comprises reacting a maleimidobenzoic acid of the formula:



with N-hydroxysuccinimide of the formula:

in an organic solvent in the presence of a dehydrating agent.

5. A process as claimed in Claim 4, wherein the reaction is carried out at room temperature for 2 to 3 hours.

6. A process as claimed in Claim 4, substantially as hereinbefore described with reference to the Example.

7. A compound as claimed in Claim 1, when prepared by a process as claimed in any one of Claims 4 to 6.

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